भारतीय मानक
Indian Standard

IS 16940 : 2018

बी.टी. कपास की दो घटनाओं, मॉन531 एवं मॉन15985 की समक्षणिक पहचान एवं विभेदीकरण के लिए पी.सी.आर. आधारित गुणात्मक पद्धति

PCR-Based Qualitative Method for Simultaneous Detection and Differentiation of Two Events of *Bt* Cotton, MON531 and MON15985

ICS 65.020

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November 2018 **Price Group 3**

Biotechnology for Food and Agriculture Sectional Committee, FAD 23

FOREWORD

This Indian Standard was adopted by the Bureau of Indian Standards, after the draft finalized by the Biotechnology for Food and Agriculture Sectional Committee had been approved by the Food and Agriculture Divisional Council.

Bt cotton is being cultivated in an area of more than 10.6 million hectares in India. *Bt* cotton events MON531 (Bollgard®I) with cry1Ac gene, MON15985 (Bollgard®II) with cry1Ac and cry2Ab genes, Event1 with synthetic cry1Ac gene, GFM-cry1A with fused cry1Ab and cry1Ac genes and MLS-9124 with synthetic cry1C gene, are being commercially cultivated.

Out of commercialized 884 hybrids of commercially released six events of *Bt* cotton, 743 hybrids (84 percent) belong to two major events, that is, 215 hybrids of MON531 and 528 hybrids of MON15985, which are being grown in farmer's fields in the North, Central, and South zones of India. Since MON15985 has an increased protection and better performance due to stacked genes amongst all the *Bt* cotton events, therefore, the yield of this cotton is comparatively higher than other *Bt* cotton events, which eventually gives higher profits to farmers. Its market price is also higher as compared to other events. But, it is difficult to distinguish the seeds of different *Bt* cotton events, so there may be chances of mixing of the events by the traders to earn profits. Hence, distinguishing these *Bt* cotton events has become an important apprehension so that the farmers may get the authentic seeds. This assay can also be employed to monitor adventitious presence of transgenes in cotton germplasm and to check for the unauthorized GM events.

To fulfill this requirement, the present provides a robust and reliable method for identification and differentiation of the two widely cultivated events of *Bt* cotton.

In the formulation of this standard, due consideration has been given to the *Food Safety and Standards Act,* 2006 and Rules framed thereunder. The standard is however subject to restrictions imposed under these rules wherever applicable.

For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the results of a test or analysis, shall be rounded off in accordance with IS 2 : 1960 'Rules for rounding off numerical values (*revised*)'. The number of significant places retained in the rounded off values shall be the same as that of the specified values in this standard.

Indian Standard

PCR-BASED QUALITATIVE METHOD FOR SIMULTANEOUS DETECTION AND DIFFERENTIATION OF TWO EVENTS OF *BT* COTTON, MON531 AND MON15985

1 SCOPE

This standard prescribes the PCR-based qualitative method for simultaneous detection and differentiation of two commercialized events of *Bt* cotton, namely, MON531 and MON15985 method intended to be used by laboratories involved in detection of genetically modified organisms and GMO derived products.

2 REFERENCES

The following standards contain provisions, which through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below.

IS No.	*Title*
IS/ISO 21569 : 2005	Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Qualitative nucleic acid based methods
IS/ISO 24276 : 2006	Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions
IS 16578 : 2018/ ISO 13495 : 2013	Foodstuffs — Principles of selection and criteria of validation for varietal identification methods using specific nucleic acid.

3 PRINCIPLE

A combination of construct and transgene-specific multiplex PCR assay in decaplex format efficiently differentiates the MON531 and MON15985 events of *Bt* cotton. Since, these two *Bt* cotton events differ from each other in their transgene constructs due to the presence of additional *cry2Ab* gene and *uidA* reporter gene in MON15985, therefore, on the basis of their different transgenic elements and constructs, a decaplex PCR method was optimized.

4 REAGENTS

4.0 General — It is generally advisable to store the reaction solutions required for the analytical method at approximately –20 °C, if not specified otherwise. It may also be appropriate to aliquot the reaction solutions required for the analytical method in order to avoid repeated freeze-thaw cycles, and/or to reduce chances of cross-contamination.

4.1 Target DNA/Amplification Control

4.1.1 MON531 DNA

4.1.2 MON15985 DNA

4.1.3 Non-GM cotton DNA (Negative amplification control for transgenic elements)

4.1.4 Samples to be screened

4.2 Test Solutions

4.2.1 Cetyl trimethyl ammonium bromide (CTAB)

4.2.2 Chloroform: Isoamyl alcohol (24:1)

4.2.3 3 M Sodium acetate

4.2.4 Absolute ethanol and 70 percent ethanol

4.2.5 RNaseA (10 mg/ml)

4.2.6 *Taq* PCR buffer

4.2.7 $MgCl_2$

4.2.8 dNTP mix

4.2.9 *Taq* DNA Polymerase

4.2.10 Hot start PCR buffer

4.2.11 Hot Start *Taq* DNA Polymerase

4.2.12 Tris base

4.2.13 Glacial acetic acid

4.2.14 EDTA

4.2.15 Metaphor agarose

4.2.16 Ethidium bromide

4.2.17 Deionized water

4.3 Primers Employed in the Assay

Target	*Primer*	*Primer sequence (5'-3')*	*Expected amplicon size (bp)*	*Reference*
cry1Ac	Cry1Ac-F/R	F-GACCGCTTACAAGGAGGGATACG R-ACGGAGGCATAGTCAGCAGGACC	228	Randhawa *et al*., 2010
cry2Ab	Cry2Ab-F/R	F-CAGCGGCGCCAACCTCTACG R-TGAACGGCGATGCACCAATGTC	260	Randhawa *et al*., 2010
aadA	AadA-1-F/R	F-TCCGCGCTGTAGAAGTCACCATTG R-CCGGCAGGCGCTCCATTG	406	Randhawa *et al.,* 2009a
nptII	APH2 short/APH2 reverse	F-CTCACCTTGCTCCTGCCCGAGA R-CGCCTTGAGCCTGGCGAACAG	215	Ding *et al*., 2004
uidA	Gus F/R	F-TTTCTTTAACTATGCCGGAATCCATC R-CACCACGGTGATATCGTCCAC	82	ISO 21569:2005
CaMV 35S promoter	SP1 F/R	F-TTGCTTTGAAGACGTGGTTG R-ATTCCATTGCCCAGCTATCT	196	Randhawa *et al.,* 2009b
nos terminator	NOS1/NOS3	F-GAATCCTGTTGCCGGTCTTG R-TTATCCTAGTTTGCGCGCTA	180	Hardegger *et al.,* 1999
Sad1	S3 F/S4 R	F-CCAAAGGAGGTGCCTGTTCA R-TTGAGGTGAGTCAGAATGTTGTTC	107	Yang *et al*., 2005
Cry1Ac transgene construct	Cry1Ac-35SF/R	F-CTTCGCAAGACCCTTCCTCTAT R-GAACTCTTCGATCCTCTGGTTG	326	Randhawa *et al*., 2010
Cry2Ab transgene construct	CTCR-F/CTCR-2R	F-ATT GAA GAA GAG TGG GAT GAC GTT A R-GAC CAG AGT TCA GGA CGG AGT T	116	Lee *et al*., 2007

5 APPARATUS

5.1 Microfuge tubes (1.5 ml)

5.2 Microfuge tubes (2.0 ml)

5.3 PCR tubes

5.4 Micropipette (100-1 000 µl)

5.5 Micropipette (20-200 µl)

5.6 Micropipette (2-20 µl)

5.7 Micropipette (0.1-2 µl)

5.8 Microtips (100-1 000 µl)

5.9 Microtips (20-200 µl)

5.10 Microtips (2-20 µl)

5.11 Microtips (0.1-2.0 µl)

5.12 Water bath

5.13 Thermal cycler

5.14 Spectrophotometer/UV-Spectrophotometer

5.15 UV gel documentation system

6 PROCEDURE

6.1 Preparation of Reagents and Buffers

6.1.1 *CTAB extraction buffer*: 2.0 g CTAB, 10.0 ml 1 M Tris *p*H-8.0, 4.0 ml 0.5 M EDTA *p*H 8.0, 28.0 ml of 5 M NaCl, 40.0 ml of H_2O. Adjust all to *p*H 5.0 with HCl and make up final volume to 100 ml with deionized water.

6.1.2 *Agarose Gel Running buffer: 50× TAE (p*H *8.0) 1 000 ml:* 2 M Tris base, 1 M Glacial acetic acid (57.1 ml), 100 ml 0.5 M EDTA solution. Weigh 242 g of Tris base and transfer to the beaker. Add deionized water to a volume of 500 ml. Mix well and add 100 ml of 0.5 M EDTA solution and 57.1 ml of glacial acetic acid. Adjust the *p*H to 8.0. Add deionized water to a volume of 1 000 ml.

6.1.3 *Preparation of 2 percent agarose gel*: Take 100 ml of 1X TAE buffer, in a conical flask. Weigh 2 g of agarose and add it to 1X TAE buffer. Mix well and boil the mixture till agarose dissolves. Cool the gel and pour it in the gel casting tray.

6.1.4 *Preparation of 4 percent metaphor agarose gel* — Take 100 ml of ice chilled 1X TAE buffer in a conical flask. Weigh 4 g of metaphor agarose and add it to 1X TAE buffer. Mix well and boil the mixture till metaphor agarose dissolves. Metaphor agarose boils vigorously, therefore do not shake the flask in between boiling. Cool the gel and pour it in the gel casting tray.

6.2 DNA Extraction and Quantification

6.2.1 Grind single cotton seed (100 mg weight) to a fine powder in approximately 500 µl of CTAB buffer.

6.2.2 Transfer CTAB/plant extract mixture to a microfuge tube.

6.2.3 Incubate the CTAB/plant extract mixture for about 1 h at 60°C in a water bath.

6.2.4 After incubation, spin the CTAB/plant extract mixture at 9 000 × g force for 5 min to spin down cell debris. Transfer the supernatant to clean microfuge tubes.

6.2.5 To each tube add 250 µl of chloroform : isoamyl alcohol and mix the solution by inversion. After mixing, spin the tubes at 9 000 × g force for 1 min.

6.2.6 Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tube.

6.2.7 To each tube add 50 µl of 3 M sodium acetate followed by 500 µl of ice cold absolute ethanol.

6.2.8 Invert the tubes slowly several times to precipitate the DNA. Generally the DNA can be seen to precipitate out of solution. Alternatively the tubes can be placed for 1 h at –20°C after the addition of ethanol to precipitate the DNA.

6.2.9 Following precipitation, the DNA can be pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate. To wash the DNA, transfer the precipitate into a microfuge tube containing 500 µl of ice cold 70 percent ethanol and slowly invert the tube.

6.2.10 After the wash, spin the DNA into a pellet by centrifuging at 15 000 × g force for 1 min. Remove all the supernatant and allow the DNA pellet to dry (approximately 15 min). Do not allow the DNA to over dry or it will be hard to redissolve.

6.2.11 Resuspend the DNA in sterile DNase-free water (approximately 50-400 µl H_2O; the amount of water needed to dissolve the DNA can vary, depending on the quantity of isolated DNA). RNaseA (10 mg/ml) can be added to the water prior to dissolving the DNA to remove any RNA in the preparation (10 µl RNaseA in 10 µl H_2O).

6.2.12 After resuspension, the DNA is incubated at 65°C for 20 min to destroy any DNases that may be present and store at 4°C.

6.2.13 Check the quality of extracted DNA using 0.8 percent agarose gels in 1× TAE buffer. Quantify the DNA by measuring UV absorbance at 260 nm and check further DNA purity based on UV absorption ratio at 260/280 nm (ranging from 1.6 to 1.9) using a UV spectrophotometer.

6.3 Preparation of Primer Dilutions

Prepare primer dilutions with deionized water to have a final concentration of 10 µM.

6.4 Singleplex PCR to Check the Amplification Efficiency of Primers

Singleplex PCR with an endogenous reference gene/chloroplast t-RNA-specific primer pair is first carried out to check for the absence of PCR inhibitors in isolated genomic DNA. If the desired amplicon is obtained, it means that the isolated genomic DNA is free from any PCR inhibitors and further PCRs can be carried out with the same genomic DNA. Amplification efficiency of all the synthesized primers is also checked using singleplex PCR.

6.4.1 Make a final volume of 25 µl PCR mixture with the following reagent concentrations:

100 ng template DNA

1X Taq PCR buffer

1.5 mM $MgCl_2$

200 µM dNTP mix

0.4 µM each of forward and reverse primers

0.5 U of *Taq* DNA polymerase

6.4.2 Perform the PCR in a programmable thermal cycler using the following the programme: one cycle of initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 1 min and extension at 72°C for 1 min; followed by a final extension at 72°C for 8 min.

6.4.3 Analyze the PCR amplicons on horizontal gel electrophoresis using 2.0 percent agarose and then visualize the results under UV light using a Gel Documentation System.

6.5 Multiplex (decaplex) PCR Targeting Construct and Transgene-specific Sequences to Identify and Differentiate Two Major *Bt* Cotton Events

6.5.1 *Preparation of 4X primer mix:* Prepare 4X concentration of primer mix by mixing 1.6 µM of each primer from the stock solutions.

6.5.2 Make a final volume of 25 µl PCR mixture with the following reagent concentrations:

175 ng of template DNA

1X Hot Start PCR buffer

3.2 mM $MgCl_2$

600 µM dNTP mix

1X primer mix

0.2 U/iL Hot Start *Taq* DNA polymerase

6.5.3 Perform the PCR in a programmable thermal cycler using the following the program: one cycle of initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 50 s, annealing at 50°C for 50 s and extension at 72°C for 50 s followed by a final extension at 72°C for 5 min.

6.5.4 Analyze the PCR amplicons on horizontal gel electrophoresis using 4.0 percent metaphor agarose gel and then visualize the results under UV light using a Gel Documentation System.

7 INTERPRETATION

7.1 Interpretation of Singleplex PCR

Singleplex PCR will be performed to check the specificity and amplification efficiency of the synthesized primer pairs. All the primers will amplify products for expected amplicon size as given in the Primer Table.

7.2 Interpretation of Decaplex PCR

In decaplex PCR (as shown in Fig. 1), for MON15985 event, all the seven inserted gene sequences, that is, *cry1Ac*, *cry2Ab*, *CaMV* 35S promoter, *nos* terminator, *uidA*, *nptII* and *aadA*, two transgene constructs, that is, *CaMV* 35S-*cry1Ac* gene construct and *ctp2*-*cry2Ab* gene construct along with an endogenous reference gene should be amplified with desired band size of 228, 260, 196, 180, 82, 215, 406, and 107 bp, respectively. In the samples of MON531 event, five inserted genes, that is, *cry1Ac, CaMV 35S* promoter*, nos* terminator, *nptII*, and *aadA* and *CaMV* 35S-*cry1Ac* gene construct along with an endogenous reference gene should be amplified with the desired amplicons of 228, 196, 180, 215, 406, and 107 bp, respectively. In non-GM cotton sample, only endogenous reference gene should be amplified and in water sample taken as negative amplification control, there should not be any gene amplification showing specificity of the developed PCR protocol.

8 NOTES

8.1 Mix the contents gently while incubation step of DNA extraction by inverting the tubes. This may minimize the shearing of DNA.

8.2 During PCR, always have a positive amplification control or/and a negative amplification control with the test samples so that a problem with the primers, enzyme or a machine setting can be ruled out.

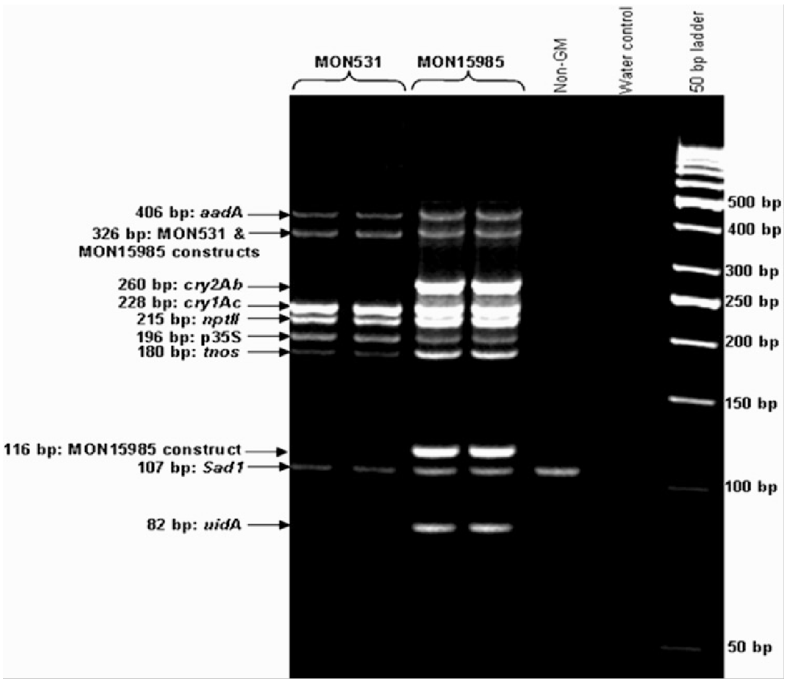

FIG. 1 EXPECTED AMPLICON SIZES OF THE TRANSGENE- AND CONSTRUCT-SPECIFIC AMPLICONS OF DECAPLEX PCR FOR DISCRIMINATION OF TWO *BT* COTTON EVENTS, THAT IS, MON531 AND MON15985

8.3 In order to make PCR assays more reliable, the target sequences and plant species specific endogenous reference genes may be detected. The endogenous reference genes are required to be included as internal control targets to assess the efficiency of PCR reactions by eliminating any false negatives. So it is always better to run PCR along with the endogenous reference gene, which would also be used as amplification control for the PCR.

8.4 As the number of primers increases in a decaplex PCR, the possible sequence-dependent interactions between primers of different primer pairs also increase, which results in the formation of primer-dimers. Small differences in amplification efficiencies for the different primer pairs might result in the preferential amplification of some of the PCR products, leaving other PCR products at sub-detectable levels. Hence, primer design, PCR cycling conditions and the concentration of each reaction component need to be cautiously optimized in order to avoid the formation of primer-dimers and to detect all DNA targets simultaneously without any primer interference.

8.5 The choice of DNA polymerase is very important for the optimum performance of the PCR. In a multiplex PCR, the Hot Start *Taq* DNA polymerase, coupled with a preoptimized primer mix for different multiplex reactions, gives the best results both in terms of reproducibility and robustness. Use of Hot Start *Taq* DNA polymerase prevents the formation of misprimed products and reduces primer-dimer formation.

ANNEX A

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This Indian Standard has been developed from Doc No.: FAD 23 (2637).

Amendments Issued Since Publication

Amend No.	Date of Issue	Text Affected

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Published by BIS, New Delhi